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14. ABSTRACT

The need to screen cerebroprotective compounds without anesthetic interference prompted development of a model using hypoxic rats. In this model two outcome measures were used: 1) the time to reach isoelectric EEG, caused by nitrogen gas inhalation in the test chamber, and 2) the time for behavioral recovery measuring the latency of restoration of the head-withdrawal reflex upon vibrissae stimulation. We report here data of blood chemistry, cerebral tissue oxygen measurements, a definition of a proposed scoring system, and the pharmacological results of RGH-2202. The findings with RGH-2202 are used here to show the utility of the screening method. Events during hypoxia: Arterial and venous pO_2, pCO_2, and pH, and brain tissue pO_2 significantly declined. Significant correlations were established among the pO_2 of cerebral tissue, blood, and the test chamber. RGH-2202 significantly and dose-dependently shortened the isoelectric EEG (iEEG) time; the compound's Effective Dose30 was 227.8 mg kg-1. Events during recovery: Full abstract see in publication.

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SCREENING FOR CEREBROPROTECTIVE AGENTS USING AN *IN VIVO* MODEL OF CEREBRAL REVERSIBLE DEPOLARIZATION IN AWAKE RATS

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The need to screen cerebroprotective compounds without anesthetic interference prompted the development of a model using hypoxic rats. In this model two outcome measures were used: (1) the time to reach isoelectric electroencephalogram (iEEG), caused by nitrogen gas inhalation in the test chamber, and (2) the time for behavioral recovery measuring the latency of restoration of the head-withdrawal reflex upon vibrissae stimulation. We report here data of blood chemistry, cerebral tissue oxygen measurements, a definition of a proposed scoring system, and the pharmacological results of RGH-2202. The findings with RGH-2202 are used here to show the utility of the screening method.

Events during hypoxia: Arterial and venous pO_2, pCO_2, and pH, and brain tissue pO_2 significantly declined. Significant correlations were established among the pO_2 of cerebral tissue, blood, and the test chamber. RGH-2202 significantly and dose-dependently shortened the iEEG time; the compound's Effective $Dose_{30}$ was 227.8 mg kg^{-1}.

Events during recovery: Immediately after the iEEG, when the atmosphere in the chamber was replaced with room air, the arterial, venous and brain tissue pO_2 increased above the control level and subsequently recovered to baseline levels. Behavioral recovery occurred before blood chemistry was otherwise normalized. RGH-2202 significantly and dose-dependently shortened the recovery time; the Effective $Dose_{30}$ was 8.71 mg kg^{-1}. The available data define and support the physiological basis of this practicable rat-screening model.   © 2001 Academic Press

KEY WORDS: pharmaceutical screening, hypoxia, isoelectric EEG, reversible depolarization, awake rat.

INTRODUCTION

Screening cerebroprotective drug candidates, promising results have been achieved in animal models of stroke; but to date, most, if not all Phase III clinical trials have failed to predict therapeutic promise [1]. It is known that various anesthetics have central nervous system (CNS), metabolic and cerebral blood flow (CBF) depressant effects [2, 3], and many compromise the predictive value of the animal models.

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To improve our chances to discover active cerebroprotective therapeutic agents, we developed an awake, freely moving rat model, which was described only briefly in preliminary publications [4–6].

A variety of parameters have been used as outcome measures of intracellular events to describe physiological insults, such as cerebral hypoxia or ischemia, and, then, to test potential drugs and other treatments to counter such trauma. However, biochemical signals such as ATP recovery require invasive tissue sampling, which makes temporal resolution difficult and inaccurate, and precludes multiple sampling from each animal. For longitudinal studies, brain electrical activity (EEG) may provide a useful signal of energy failure and of recovery
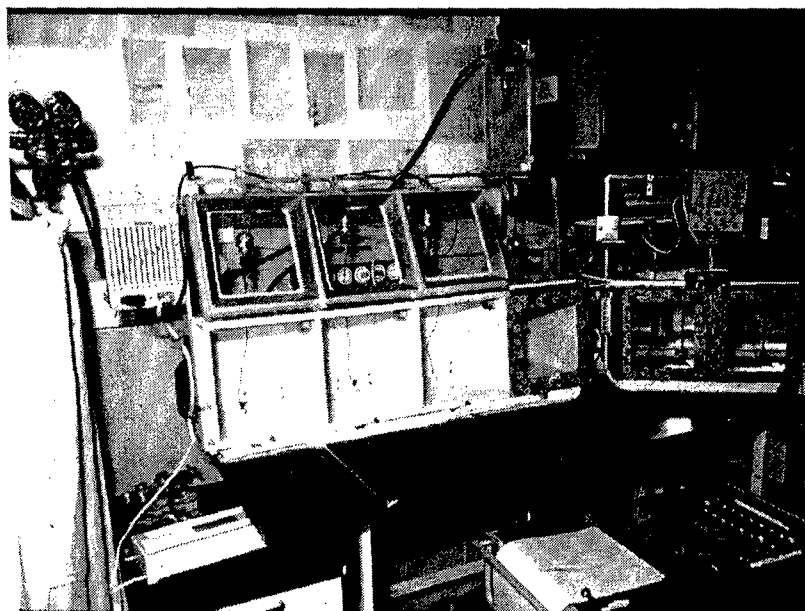
Fig. 1. Site of iEEG measurement. On the left side are nitrogen tanks with the pressure regulator. The gas flows from the tank into the automatic flow control unit, and then into hypoxic chambers via arborized silicone tubes of equal length. The gas control unit is located on the right upper corner of the picture, next to the 6-channel scope on which the EEG was also monitored. Hypoxic chambers are placed on the table in two units: in front four, and to the right, a double. Nitrogen inflow holes are located on the rear lower part of the chamber. In the front, lower right corner the recording, EEG 8-ch equipment is visible. Each chamber is equipped with a Plexiglas front door (the three on the left are open); swivel connectors are suspended above the chamber with EEG connecting cable (shielded), allowing free movement of the rat. Nitrogen outflow is on the middle of the top panel, with an EEG connection wire going to the swivel connector. In the middle of the chamber (lower edge) is the micro switch, operated by opening the door. This switch indicates the end of the iEEG period and, at the same time, the start of the recovery time. In the right upper corner of each chamber is the switch, protected by an aluminum shield, used for signaling the end of recovery time. Each door has an isolation foam strip (1.5 cm width) on its edge and an aluminum sheet (5 cm width) fixed to the door edge around the door. This remote barrier prevented the rat from obtaining extra oxygen from potentially damaged foam. The swivel connectors are connected to the switchbox for all six rats (left, between the gas tank and first box from the left). During the life and use of a rat, the head connector pins are often damaged. It was practical to implant more than three electrodes and, with the help of a switchboard, to select the working connector pins, i.e. to save the rat for further measurements.

afterward [7–12]. This may be especially true in freely moving animals since the effect of anesthesia would not compromise the study's outcome measures.

The aims of this report were: (1) to define an *in vivo* animal model used to screen for the effects of drugs on cerebral hypoxia and recovery afterward; and (2) to describe the hypoxia-induced pathophysiology and elaborate the theoretical and practical background of this model. For illustration of a drug result, results with RGH-2202 (L-6-ketopiperidine-2-carbonyl-leucyl-L-propin-amidine) are presented. Preliminary reports of data for pharmacology, CBF, lipid peroxidation and electroanalytical measurements of this animal model have been introduced elsewhere [4–6, 13].

MATERIALS AND METHODS

EEG measurements were recorded from awake male Wistar rats ($n = 6$ per group) placed individually in a test chamber (Fig. 1) ventilated with room air ($pO_2 = 20.92$ v/v%). Hypoxia was induced by 100% nitrogen inflow with a flow rate of $1.67 \ l \ min^{-1}$ [4].

Measurement of isoelectric EEG (iEEG)

These studies emphasized two outcome variables. One outcome variable is the 'iEEG-time', and the other is the 'recovery time'. The iEEG-time is the period from the onset of nitrogen inflow to the first appearance of a two-second flat line of EEG (Fig. 2), after which the door of the chamber was opened to restore normal environmental oxygen level. The second outcome variable was 'behavioral recovery' designated as the time that elapsed between the opening of the chamber's door and the first spontaneous or reactive movement of the rat induced by vibrissae stimulation provoking a head-withdrawal reflex [14].

For each animal, an initial control EEG recording was taken two weeks after electrode implantation, and regularly thereafter (at least twice per year) to maintain the implanted animal test cohort. In a typical screening sequence, EEG recordings from each of six rats were made continuously and simultaneously prior to the onset of hypoxia and throughout the period of nitrogen-induced hypoxia, until the behavioral recovery outcome was established. In the routine of screening, each rat had two days of rest (i.e. without testing) between test episodes. The oxygen concentration in each chamber was measured by an oxygen monitor (OM-15, Beckman, CA) in the presence of the animal. The initial (baseline) test chamber pO_2 was 20.9 v/v% ($n = 10$); during iEEG-time it decreased to 2.96 ± 0.38 (mean and SD, $n = 4$).
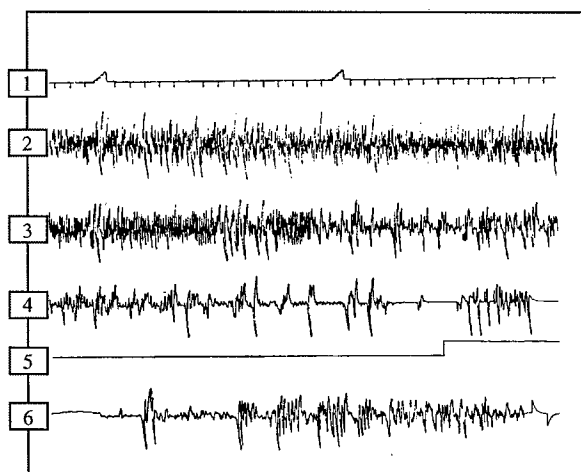
Fig. 2. EEG recording during hypoxia and recovery. Upper trace (1) is the time-marker with second divisions. Four consecutive epochs of EEG trace (lasting 35 s each) are placed on the figure starting with the upper trace. The trace (2) shows the baseline (before hypoxia). Trace (3) shows the first alteration (decreased incidence of regular, theta-delta waves) in EEG a few minutes before iEEG. During trace (4) EEG became isoelectric several times. On trace (5), an upward pen deflection indicates the opening of the door of the box, at the end of the hypoxic period and at the start of the measurement of recovery. The restoration of EEG is shown on trace (6), with a real time scale, (without a missing period). The EEG calibration is located in the lower right corner: amplitude 100 μV, high frequency filter 70 Hz, time constant 0.3 s.

The time to reach the two outcome (variables) was measured by means of an IBM-compatible PC configured to measure the two separate time intervals for each animal chamber, and with software incorporated into a dBase program.

Animal environment, housing, management, drug storage and administration were maintained according to appropriate laboratory practices [15]. For record-keeping purposes, a standardized record book on laboratory practice was maintained covering neurophysiological preparation, screening procedure, and administration of results [15–17].

The number of measurements for the screening procedure (by year) was as follows: 1987, 540 measurements; 1988, 857; 1989, 694; 1990, 602; 1991, 823; 1992, 987. These numbers involve the routine screening, the drug-free control and ED_{30} measurements. The number of measurements was from 3–4 groups/die. The standard was six rats per group. The laboratory personnel were one supervisor, one researcher and two technicians.

In addition to the data from freely moving animals, we here also report, for comparison, results from rats under anesthesia (pentobarbital, 60 mg kg^{-1}, i.p.).

Measurement of brain tissue oxygen tension

Brain tissue oxygen tension (pO_2) was monitored by a Clark electrode [18], detailed elsewhere [5], and with a polarographic electrode [18] implanted into the cerebral hemisphere ($n = 25$ trials; $n = 12$ rats) in the same manner described previously [4]. For comparisons among animals, the polarographic currents were normalized to baseline, defined as: pO_2 level prior to hypoxia. It was designated as 100% and all subsequent pO_2 values are expressed as a percentage of the pre-hypoxic baseline.

Measurement of blood chemistry

The variables measured were: blood pH, pO_2, and CO_2, and hemoglobin.

Measurements were made with an ABL-3 analyzer (Radiometer, Denmark). Blood samples from awake animals were collected from the ophthalmic venous plexus by positioning the blood sample tube at the inner angle of the eye [19]. Blood was sampled immediately before the onset of hypoxia, during the iEEG-time, and at the time of behavioral recovery. Blood samples from anesthetized animals were taken via catheterization from the aorta abdominalis and vena iliaca. Sampling was done immediately before the onset of hypoxia, in 10 min intervals during hypoxia until the iEEG-time, and in 5 min intervals during the behavioral recovery phase.

In addition, blood glucose levels were determined in samples taken from the ophthalmic venous plexus [19] from groups of awake rats (fed or fasted for 24 hours; $n = 23$), by the glucose dehydrogenase method (Centrifugal Analyzer, Electro-Nucleonics, Inc. USA).

Measurement of cerebral blood flow

CBF changes during hypoxia were recorded by hydrogen clearance and electrochemically generated hydrogen and reported in detail previously [4, 13]. Furthermore, CBF was recorded with a PeriFlux PF 3 laser Doppler perfusion monitor and PF 408 standard probe (Perimed, Sweden). In anesthetized rats ($n = 9$), measurement of epidural CBF was made immediately after implantation of the probe; the procedure was repeated in awake rats several days later ($n = 12$).

Drug administration

In the routine screening procedure, all drugs were administered orally using a rigid intragastric dosing needle [19]. Six rats per group received drugs 30 min prior to the onset of hypoxia. The test compounds were most often dissolved in distilled water; in the event of poor solubility, the compound was dissolved in chremophore (2.5 mg kg^{-1}). A dosage of 50 mg kg^{-1} p.o. was used as the initial routine screening dose. Subsequently, for known drugs or effective compounds, various dosage levels were subsequently assessed. For each dose level, data from 12 to 24 rats were used.

Scoring

iEEG-time. The applied scoring system is known in pharmacology as probit analysis for dose-response or dose-effect relationship [20, 21]. For pharmacological comparisons in routine screening, to measure and compare the effectiveness of a screened drug, the

Table I
Summary statistics of baseline data

Variable	iEEG-time	Behavioral recovery
Sample size	1231	1231
Average	27.1398	4.59079
Median	27	4.42
Mode	26	4
Geometric mean	26.9647	4.40977
Variance	9.47989	1.86603
Standard deviation	3.07894	1.36603
Standard error	0.0877551	0.0389341
Minimum	18	1
Maximum	39.77	15.12
Range	21.77	14.12
Upper quartile	29	5.12
Lower quartile	25.02	3.99
Internal quartile range	3.98	1.13
Skewness	0.194348	1.96656
Standardized skewness	2.78377	28.1682
Kurtosis	0.424076	10.7002
Standardized kurtosis	3.03715	26.6332

The data of the above variables were subdivided into 31 classes.

iEEG-time was scored according to the following formula:

$$\frac{|t|c|t|/c}{}$$
$$\text{effect}\% = \text{sgn}(t)10 - 1$$

where
sgn(t) is the sign (positive or negative) of the number (t).
t = Difference in iEEG-time (absolute value in minutes) vs control value; c = 45.23: a normalization factor calculated on the basis of control mean value.

— Baseline iEEG-time in control animals = 27.13 min (Table I).

— 40.69 min (150% control mean value) equals +1, i.e. 100% effect.

— Effect is an exponential (base-10 logarithm) function of time.

For every typical screened drug, the average iEEG-time (% effect) was determined using data from six rats; a test compound having a score higher than 15% was considered 'effective' in the first pass, routine screening.

Behavioral recovery time. The average recovery time (ART) was calculated from data obtained from the same six rats as part of the screening paradigm.

ART was expressed as a percentage of the baseline (drug- & placebo-free control) value.
The formula was the following:

$$x = (100^*y)/c$$

where
x = recovery score; y = time of recovery (in minutes); c = constant, average recovery time of control group, 4.59 (Table I).

A compound having a score higher than 15% was considered 'effective' in routine screening.

Table II
Squared correlation coefficients (R^2) among different pO_2 modalities during hypoxia

Modality 1	Modality 2	R^2
Arterial anesthetized	Box air	0.9385
Arterial anesthetized	Polarographic anesthetized	0.9364
Arterial anesthetized	Polarographic awake	0.9146
Arterial anesthetized	Polarographic awake	0.9996
Arterial anesthetized	Polarographic anesthetized	0.9984
Chamber air	Clark anesthetized	0.9873
Chamber air	Polarographic anesthetized	0.9866
Chamber air	Clark awake	0.9865
Chamber air	Polarographic awake	0.9825
Clark anesthetized	Polarographic anesthetized	0.9998
Clark anesthetized	Clark awake	0.9991
Clark anesthetized	Polarographic awake	0.9890
Polarographic anesthetized	Polarographic awake	0.9874

Data were analyzed only before iEEG period. Clark: Clark electrode, polarographic: polarographic electrode. Rats were in groups of Anesthetized or Awake.

Effective dose$_{30}$. To establish a dose-response curve, an effective dose (ED_{30}) was subsequently calculated from a linear regression program (see below [20, 21]).

An effective dose (ED_{30}) value, defined as the dose level causing a 30% difference from control, was determined for both iEEG and behavioral recovery time, if the regression was statistically significant. A paired t-test and two-way ANOVA were used for statistical analyses. A value of $P < 0.05$ was considered significant. The confidence interval was 95%. For ED_{30} calculation, the self-control (drug- & placebo-free) data were used. The formula was the following:

$$\log Y = B0 + B1^* \log X$$

where
$\log Y$ = log measured − log control; $B0$ = intercept; $B1$ = slope; $\log X$ = log dose.

Histological study
For histological control, with ironhematoxylin/eosine staining, the brains and hearts of eight rats after hypoxia ($n = 4$ awake, and $n = 4$ anesthetized) were observed under light microscopy (10–20 μm cuttings).

RESULTS

Events during hypoxia
iEEG-time. When nitrogen was used to induce hypoxia in the test animal chamber, within approximately 25 min, the oxygen concentration in the chamber decreased to 2.96 volume%.

In control (drug- and placebo-free) animals, the average iEEG-time was 27.13 min. This time was used as the baseline or 'control' value for routine screening (see earlier calculation of effect percentage). The average iEEG-time of 1231 animals resulted in a Gaussian
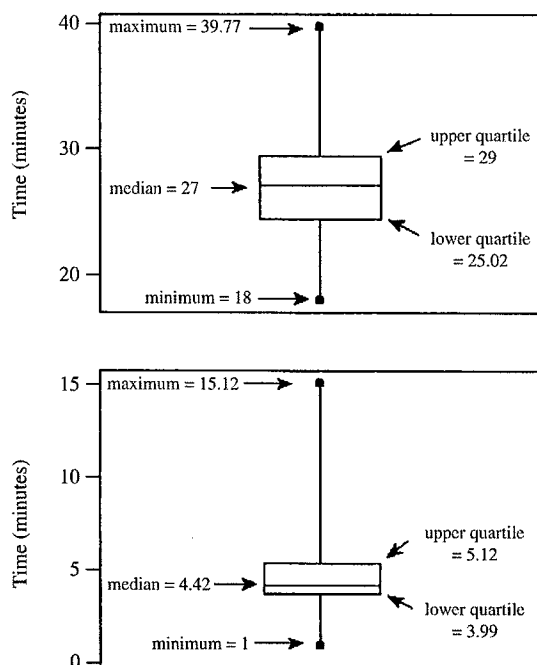
Fig. 3. Five-value summary plot of iEEG-time (upper part) and behavioral recovery time (lower part). The boxplots are characterizing the distribution of data derived from the control measurements of 1231 rats. The data of iEEG-time shows symmetric (Gaussian) distribution; the behavioral recovery is non-Gaussian. Further details are in Table I.

distribution, supported by the following (Fig. 3; for details see Table I):

(a) Mean iEEG-time (27.1 min) was close to the median (27.0 min).

(b) Lower and upper quartiles were equidistant from the median.

(c) Minimum and maximum were approximately equidistant from the median.

(d) Skewness was near zero (i.e. a symmetric distribution).

(e) Kurtosis was near zero (i.e. appropriate peakedness).

(f) Frequency histogram was normally distributed.

(g) Standardized lower and upper quartiles were close to the corresponding theoretical z-values.

Brain oxygen levels. Brain tissue pO_2 continuously decreased during hypoxia until the EEG become isoelectric, i.e. the iEEG (Table III and Fig. 4). Correlation coefficients during hypoxia among differently measured oxygen levels showed a strong and significant correlation (Table II). The baseline value of brain pO_2 in awake and anesthetized groups differed significantly ($P = 0.00084$); other details are summarized in Table III.
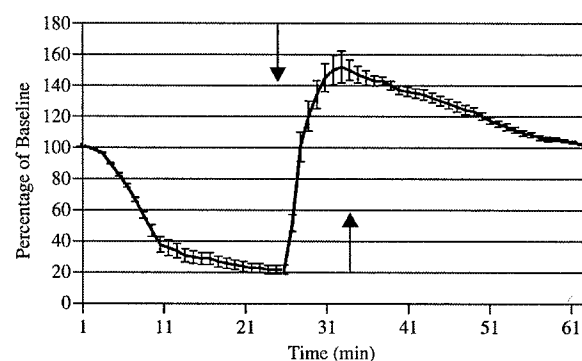

Fig. 4. The effect of hypoxia on the dynamics of brain tissue pO_2 levels in anesthetized rats. Data are shown as a percentage of the baseline (mean). Vertical bars show the ±SEM. Brain tissue pO_2 was measured by polarographic electrode ($n = 25$). The appearance of isoelectric EEG is indicated by ↓, and the typical recovery time by ↑. In anesthetized rats there was no behavioral recovery at this time, but in both cases, the pO_2 similarly did not recover to baseline level.

Blood chemistry. In awake animals blood chemistry changes during hypoxia were observed and compared to baseline data (before hypoxia). Hemoglobin content did not change; pH significantly decreased; and arterial pH and pCO_2 decreased.

For comparison, in anesthetized animals the arterial oxygen content showed a significant ($P < 0.0001$) decrease only during iEEG-time; these results are summarized in Table III.

The awake and anesthetized venous variables were significantly different in baseline pH ($P < 0.00001$) and in pO_2 during iEEG time ($P < 0.01$; Table III). The blood derivative data followed the change of their main determinants; in most cases they were decreased both in awake and anesthetized groups.

Fasted animals had significantly lower baseline blood glucose levels than fed controls (75.25 and 215.47 mg dl^{-1}, respectively; $P < 0.01$). During hypoxia, blood glucose level decreased markedly in fed animals, and there was a significant decrease in iEEG-time (29.5 min for normoglycemic, and 25.6 min for hypoglycemic, respectively; $P < 0.001$). Fasted animals had significantly lower blood glucose levels during iEEG-time than fed controls (84.25 and 132.82 mg dl^{-1}, respectively; $P < 0.001$).

Cerebral blood flow. In the present study the CBF during hypoxia in anesthetized animals was lower when compared to that of awake controls. When anesthetized animals were made hypoxic immediately after the implantation of electrodes, at the time of EEG suppression, CBF was decreased (mean = −38.1% of baseline). In contrast, CBF rose significantly ($P < 0.0001$; mean = 58.7% of baseline) during iEEG, when the animals were made hypoxic without anesthesia. The inter-individual difference was not significant ($P = 0.8649$). Preliminary data reporting CBF changes were provided elsewhere in detail [4].

Table III
The brain tissue pO_2 and blood chemistry data during the times of baseline, iEEG-time, and behavioral recovery

	Unit	Baseline	±SD	n	iEEG	±SD	P	n	Recovery	±SD	P	n
						Awake						
Brain	mmHg	42	0.02	3	5.34	0.21	0.0001	3	79.13	1.79	NS	3
Venous												
pH		7.31	0.1	30	7.15	0.18	0.001	12	7.06	0.15	0.001	7
pO_2	mmHg	40	6	30	56	20	NS	12	67	13	NS	7
pCO_2	mmHg	53	6	30	27.5	7.5	0.0001	12	28	3.5	0.001	7
						Anesthetized						
Brain	mmHg	38	1.2	6	6.4	6.4	0.0001	6	56	22.2	NS	6
Arterial												
pH		7.03	0.05	9	7.03	0.2	NS	5	7.03	0.25	NS	4
pO_2	mmHg	110.16	27.5	9	32.78	20	0.0001	5	133.25	22	NS	4
pCO_2	mmHg	66	21	9	60	8	NS	5	57.5	8	NS	4
						Anesthetized(cont)						
Venous												
pH		7.09	0.1	7	7.08	0.06	NS	4	7.07	0.07	NS	3
pO_2	mmHg	36.2	11	7	21.58	22	NS	4	46.73	21	NS	3
pCO_2	mmHg	54	14	7	47	33	NS	4	38	10	NS	3

In *Awake* rats all data represent venous values, as the sample was taken from the ophthalmic plexus. Under *Anesthetized rats* there are arterial and venous data. The brain pO_2 was measured by electroanalytical method (Clark electrode). In *Anesthetized rats* there was no recovery, so the value is given 5 min after the iEEG-time (the time of behavioral recovery of awake rat). Test of significance was calculated between baseline and iEEG-time or behavioral recovery values (two-sample *t*-test is assuming equal variances; Minitab software was used). P = Significance, related to baseline (before hypoxia) of the same group; NS = not significant, n = number of rats.

Recovery after hypoxia

Recovery time. Within a few seconds after reoxygenation (i.e. opening the door of the animal's chamber) the EEG recovered (Fig. 2). The average baseline behavioral recovery time was 4.6 min. These data served as a control value for routine screening studies. The recovery time resulted in a non-Gaussian distribution, supported by the following (Fig. 3, also Table I):

(a) Minimum and maximum were not equidistant from the median value.

(b) Skewness was not close to zero (i.e. not symmetric, but positively skewed).

(c) Kurtosis was not close to zero (i.e. distribution too peaked to be normal).

(d) Frequency histogram showed a distribution skewed to the right.

Blood and brain oxygen levels. Immediately following reoxygenation, the blood and brain oxygen levels increased above the control levels (Table III). Within approximately 30 min, blood and brain pO_2 returned to approximately control levels (Fig. 4).

Recovery time and glucose levels. Recovery time for hypoglycemic rats was 4.6 min; recovery time for euglycemic rats was 5.3 min; these values differed significantly ($P < 0.05$). As expected, fasted 24 h-animals had significantly lower blood glucose levels than fed control animals (85.3 and 101.8 mg dl^{-1}, respectively; $P < 0.01$).

Other results

There were no resulting histological differences in the brain/heart tissues of awake and anesthetized rats. Furthermore, there were no detectable light microscopic changes caused by hypoxia.

RGH-2202 (a TRH analogue) significantly shortened the duration of iEEG-time as well as the time to behavioral recovery. Both outcome variables were dose-dependent and statistically significant. The ED_{30} for iEEG-times (with 90 min of pretreatment, because of low solubility and absorption) was 227.80 mg kg^{-1} p.o. (CI: 137.650–376.96 and $t = -4.187$, $P < 0.05$). For recovery times, the ED_{30} was 8.71 mg kg^{-1} p.o. (CI: 3.15–24.03; $t = 2.068$, $P < 0.05$; Fig. 5).

DISCUSSION

Theoretical considerations

This paper describes a high-capacity screening test designed to identify cerebroprotective compounds without anesthetic interference. The authors detail the pathophysiology of the hypoxia-induced events, and theoretical and applicable background information. Also described herein are ascribed scoring systems of the screening model used, and confirmation of the model's reversible nature, supported by data of histology and metabolism, and a preliminary report (6).

Others have used animal test models indexing protection against lethality; however, the main conceptual difficulty is to define the 'lethal threshold'. In contrast, the proposed outcome variable 'iEEG-time' is a practical method (i.e. to measure 'the first appearance of objective
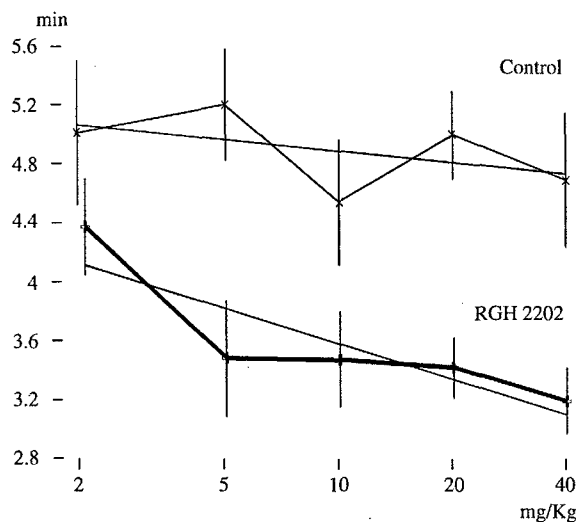
Fig. 5. Changes of recovery time under the effect of RGH-2202. The drug significantly shortened the recovery time. The administration was performed i.v., into the tail vein immediately at the time of the appearance of isoelectric EEG. Data are mean $\pm$ SEM, $n = 12$ in each group. The ED_{30} was 8.71 mg kg^{-1} (CI: 3.15–24.03; $t = 2.068$, $P < 0.05$).

irreversibility' [9, 22–27]). The measurement of brain DC potential or extracellular K^+ is also a potential indicator of the earliest, possible reversible changes, as was demonstrated by reversible depolarization [28, 29]. The application of these latter methods has practical drawbacks compared to iEEG, however when applied to large capacity pharmacological screening procedures for neuroprotective agents.

According to Irwin: 'An intact animal that has received no medication must be used whenever possible in evaluating a drug's activity' [30]. Although this is the ideal goal, the reality is that researchers often need to select an anesthetic technique that interferes as little as possible with the particular experiment [31]. It is known that anesthetics have distinct influences on cerebral metabolism and CBF [2, 3]. The most plausible solution to exclude any interference is to use no anesthesia at all, while pharmacological screening is performed on awake, freely moving animals.

Another problem is whether the test challenge allows for the estimation of reversibility. The iEEG model employed in this study demonstrated reversibility by various methods. The physiological character of this model was confirmed by our analysis of lipid peroxidation, which indicated there was no neuronal membrane damage as a result of applied hypoxia [6]. These findings are supported by the recent data of blood chemistry and histological data. The implications are that interference due to anoxic depolarization [12], robust changes in brain redox environment [32], accelerated apoptosis or late injury may be excluded [26, 33].

The best neurophysiological descriptive characteristic of this model should be described as 'reversible depolarization', rather than irreversible anoxic

depolarization, the latter of which is observed with severe damage, such as characteristic in the core region of ischemic damage [7]. The basis of this brain status (reversibility) is the shortness of the insult, guided by iEEG, the resulting transient brain tissue oxygen decrease, and a compensatory CBF increase indicative of the intactness of CBF autoregulation [4, 34]. A further CBF increase, commonly observed in screening models using asphyxia caused by high CO_2 concentration, was not observed when using this relative short exposure to nitrogen and spontaneous respiration. In regard to CBF data, the model described herein seems to be a penumbra-like model [7]. It is based on iEEG orientation supported by clinical experience related to the iEEG and recovery time [8]. The influence of anesthesia on CBF and its reactivity to hypoxia was demonstrated earlier [4] as well as in the present study.

The difference of events observed during either the hypoxia and recovery test cycles was appropriately characterized by blood chemistry data and neurotransmitter (chatecolamine and serotonin derivatives) release, described elsewhere [5] and further corroborates the findings of Cohen [35]. This metabolic difference also correlates to the different scoring formulas used herein. During hypoxia, a better approximation was given using exponential (10-based logarithm) curve fitting, while the recovery was described with a linear formula (i.e. percentage calculation). This dual status of brain metabolism was possibly reflected in the characteristic effective dosages of the same compound (RGH-2202).

We hypothesize that surviving hypoxia is characterized by an exponential function of time rather than a linear one, and this is expressed by the scoring of the iEEG-time (modified probit analysis) [37–39].

Practical considerations

One of the first data outputs obtained during the development of this animal screening model was the spectral analysis of the EEG (sEEG). The sEEG endpoint was rejected as a screening output after preliminary experiments showed no practical value of the spectral metrics of EEG cessation in characterizing of the pharmacological agents screened. Rather, the more objective endpoint of isoelectricity, defined as the iEEG being the first appearance of a 2-second flat line of EEG (modified Grade 4 by Prior [8]), was introduced and adopted as one of our outcome variables. In evaluating the EEG recovery time, the rapid restoration of the EEG activity in the rat made it a non-specific indicator of recovery; therefore, the return of the spontaneous orientation reflex measuring the latency of behavioral restoration of the head-withdrawal reflex induced by vibrissae stimulation was evaluated [8, 14] and adopted as our second outcome variable.

The main goal of this report was to share our understanding of the oxygen-related *in vivo* phenomena during hypoxia and understand better the biological substrates upon which we screen for new active cerebroprotective therapeutic agents. The importance

of blood glucose level, as a hypoxia-'compensating' factor has been described previously [40, 41]. The thesis demonstrating shorter iEEG and behavioral recovery time in hypoglycemic animals is consistent with the current findings, expressed as a longer iEEG time in normoglycemic rats. The prolongation of behavioral recovery time in the same group also correlates with Siesjo's finding [40, 41] and reflects the difficulty of the animal's ability to eliminate the increased volume of lactate, when compared to that in hypoglycemic rats.

Computerization of the outcome measurements offers the possibility of data 'magnification'. The virtual enlargement of the time scale (in other words, detect the physiological events in a second-base scale) around cerebral death could be beneficial in the intraoperative and postoperative intensive care monitoring. In clinical practice, the therapeutic time window in cases of acute ischemic stroke is 3 h [42, 43]. Recent new therapeutic possibilities regarding inhibition of inflammatory mediators, underscores the importance of early, minute pathophysiological changes [22, 44–51].

During the seven-year period this screening procedure was operative and active, the iEEG-time of the control animals remained remarkably consistent. There was no habituation to hypoxia during repeated use of the same members of the rat colony; 'beginner', 'trained control', or aged rats showed comparable control-like results. However, the control iEEG-time was affected by certain variables such as a change in iEEG time in response to increased room temperature of the vivarium or with concomitant illness (e.g. pneumonia). These findings are explained by the increased basic metabolism and in the second example, by the decreased surface area in lungs (verified by necropsy). When such confounding issues were revealed, the routine screening was halted and the causes were eliminated before resuming the screening procedure.

Hypoxic period

The present iEEG model is hypoxemic hypoxia and deficient tissue oxygenation is a result of inadequate arterial oxygenation [18]. During hypoxia the available hemoglobin is insufficiently oxygenated and the resulting oxygen content is inadequate to maintain an adequate blood-to-brain tissue oxygen tension gradient (brain pO_2). Hypoxia and/or ischemia form a positive feedback loop [9], where a cascade of biochemical events takes place. For example, during hypoxia, acidosis develops because of anaerobic glycolysis, which starts at about pO_2 35 mmHg. This results in an accumulation of lactic acid and a decrease in pH (metabolic acidosis). During acidosis, brain energy decreases, and the brain is more prone to edema formation. In acidosis, the dissociation curve of oxygen shifts to the right [18]; at the same level of pO_2, the saturation is decreased. During hypoxia or ischemia, the brain's energy saving strategy is essential. It is possible that cessation of electrical activity (the iEEG) is an important protective process [52, 53].

The direct measurement of brain tissue pO_2 demonstrated a tight correlation to arterial and venous pO_2 in this model, which is consistent with the observation that the brain venous pO_2 begins to approximate tissue pO_2 as arterial pO_2 declines [54, 55]. The brain tissue pO_2 reached the level of critical oxygen tension (5.34 mmHg in awake rats, and 6.4 mmHg in anesthetized rats) [56] only at the time of iEEG (i.e. 2 s). This is the reason why oxidative metabolism and the consequent ATP production were stopped for a very short, transient period. The cessation of ion transport was limited to two seconds (i.e. isoelectric EEG). This is the key factor associated with reversibility (i.e. reversible depolarization).

In the iEEG model, transient, partly compensated metabolic acidosis [18] was observed, and this was expressed by decreased pH, pCO_2, and HCO_3.

Both the small pH change during anesthesia and the robust decrease that occurred during the awake state in this animal model might be related to the difference in lactic acid accumulation [57].

The rat model described here has revealed similar stages of hypoxia as described by Dunn *et al.* [55]; specifically, lactic acidosis, fall of pH, decrease of ATP, profound acidosis and isoelectric EEG. The compensation for hypoxia, mediated via carotid and aortic bodies, as well as peripheral chemoreceptors, involves various cardiopulmonary compensatory systems (increased cardiac output, oxygen extraction, blood buffers (HCO_3, respiration), and temperature regulation. The role of phosphate and ammonia buffers (mediated via kidneys) in the iEEG model was limited, since the time required significantly affected blood pH in hours, in contrast to respiratory mechanisms, which can change significantly in seconds [18].

The effect of hypoxia in the awake rat was slower when compared to the typical response in other models, but was intense enough to trigger all physiological protective mechanisms. Although compensations to hypoxia have different speeds of reaction, they are functionally related to each other. The cardiac and respiratory compensations of hypoxia, such as hyperventilation, and possibly increased heart rate were typically present, but unmeasured in these experiments. A future study is needed to reveal the potential role of these factors.

Recovery period

The mechanisms of reperfusion damage following focal cerebral ischemia/hypoxia have not been described in detail. Recent results, however, strongly suggest that reactive oxygen species, generated during the reperfusion period, may trigger the reperfusion injury [40, 58].

As designed, at the end of the experimental hypoxia (during iEEG-time) the rat lost consciousness. Opening the door of the hypoxic animal chamber was the start of the recovery measurement. The reflex of the first spontaneous or reactive movement of the rat, following vibrissae stimulation, is called 'head-withdrawal reflex' [14]. This offered a practical indicator of recovery of spontaneous

orientation, reflecting operative and integrated CNS activity and restored cognitive functions. It has been suggested that the time to behavioral recovery may reflect the elimination of free radicals and neurotoxins [59, 60].

In this iEEG model there was dissociation between the restoration of EEG, behavioral restoration, and the metabolic status [35]. Accordingly, the EEG returned to normal within seconds. Recovery of behavior and CBF occurred within 5 min [4], while metabolism (blood chemistry) and brain tissue pO_2 required at least 20 min to recover. A similar time range was shown for recovery of pH regulation [61] and for blood glucose [62].

Pharmacological aspects

The pharmaco-economic and animal protective aspects of this screening model are out of the scope of this discussion; therefore we can only refer to some related publications [63–70].

Neuroprotective agents are wishfully being designed to work either during acute ischemia or during reperfusion, when additional brain injury may occur. Despite the completion of a number of clinical trials investigating neuroprotective agents that have various mechanisms of action, no effective agent has been identified [71]. In the case of stroke, cerebroprotective drugs could interrupt the ischemic cascade in tissue that is not yet dead (i.e. their effect is primarily on the penumbral region [28, 42]). The iEEG model, because of its reversible, penumbra-like nature, might serve as a practical screening model for neuroprotective compounds.

A fundamental difference was observed between the results presented here, and those of others reporting on cerebroprotective compounds measured by EEG. The present effect is expressed as a shortened time to isoelectricity [4]. We concluded the difference could be explained by the absence of anesthesia in our model. The differences in many of the measured variables were significantly different between awake and anesthetized animals.

We hypothesize that a prolonged iEEG might afford the animal more 'resistance' to the hypoxia; however, a decrease in iEEG-time caused by an effective cerebroprotective drug might reflect decreased brain metabolism to save energy, as discussed earlier [4]. This is consistent with the therapeutic goal: that a major effect of the drug is exerted on the recovery of energy metabolism of the tissue, since recovery reduces a secondary deterioration in the bioenergetic state [40]. The recovery time measurement in our iEEG screening model is believed to represent this process.

In clinical situations, such as postoperative recovery period, a decrease in behavioral recovery time as an outcome is desirable. In our model, the recovery time measurement was a valuable outcome.

The thyrotropin-releasing hormone (TRH) has been postulated to be involved in the regulation of seizures and neural degeneration. For example, the results obtained with the TRH analogue drug, RGH-2202 (L-6-ketopiperidine-2-carbonyl-leucyl-L-prolin-amide), were targeted to accelerate the postoperative recovery of patients from unconsciousness [72]. This observation is consistent with other neurophysiological observations of the effect of RGH-2202 [73, 74].

The ideal protection of neural tissue may be produced by a combination of drugs rather than a single agent [64, 76]. Putative drugs that act at several different levels in the ischemic cascade may be likewise more effective than those with a single mechanism of action [43]. The application of this multimodal approach was the background of our screening model (i.e. to record the parallel or related changes during the iEEG measurement in order to reveal related and potentially important factors). This goal is consistent with the expectation that measures of functional improvement should be incorporated to characterize neuroprotection [77, 78].

The main advantages of this iEEG model are its simplicity and its high screening capacity. The results of this test were integrated into a scoring system, involving other biochemical, psychopharmacological, and toxicological tests. Furthermore, the results were supportive in the design of molecular structure: the results of iEEG screening allowed the chemist to create a series of new molecules as a function of iEEG score [79]. It was used in the collaboration of Takeda C. I. Ltd., (Japan) and G. Richter Ltd., (Hungary) for Anti-Dementia Drug Research [17]. Richter Ltd. registration number is: RGD 42956.

The introduced iEEG model could be used as a sensitive neurotoxicity test as well [80, 81].

CONCLUSION

The iEEG model of the unanesthetized, freely moving rat represents a relatively high capacity and efficient *in vivo* model for screening neuroprotective drugs. In this model the pharmacokinetics and pharmacodynamics are without potentiation or interaction caused by anesthesia. In this iEEG model, all physiological homeostatic and compensatory mechanisms were present and intact. Repeated use of the same animal is life and cost saving; repeated testing caused no apparent artifacts in the screening tool. Evaluations to compare animal and clinical results [1, 71, 77, 82, 83], as well as pharmaco-economical and animal protective regulations, support this approach.

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